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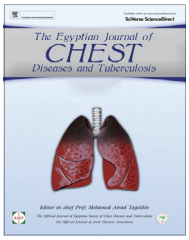

ORIGINAL ARTICLE

Detection of fluoroquinolone resistance in *Mycobacterium tuberculosis* clinical isolates as determined by gyrA/B gene mutation by using PCR technique

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KEYWORDS

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Abstract Fluoroquinolones are broad-spectrum antimicrobial agents that have been used with increasing frequency over the past decade.

Fluoroquinolones have in vitro and in vivo activity against *Mycobacterium tuberculosis*.

However, resistance to fluoroquinolones in cases of tuberculosis is not routinely assessed.

Mutations in a small region of gyrA, called quinolone resistance-determining region (QRDR) and, less frequently, in gyrB are the primary mechanism of FQ resistance in *M. tuberculosis*.

PCR-based techniques provide new possibilities for the rapid diagnosis of first- and second-line drug resistance.

Results: There were 40 consecutive adults, who had culture confirmed pulmonary tuberculosis during the study period.

Mutations were observed in the QRDRs of both gyrA and gyrB in 22 isolates (55%). Only gyrA +ve in 7(17.5%) isolates. Only gyrB +ve in 5(12.5%) isolates. Total gyrA +ve in 29(72.5%) and total gyrB +ve in 28(70%) isolates. Both gyrA and gyrB −ve in 6 (15%).

Conclusion: The incidence of FO-resistant *M. tuberculosis* is gradually increasing to alarming levels this may be due to wide spread use of this vital groups of drugs in community-acquired pneumonia and urinary tract infections.

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Introduction

The World Health Organization (WHO) estimated that there were 0.5 million cases of multi-drug resistant (MDR) tuberculosis (TB) in 2007. Only 8.5% of the estimated global total of smear-positive cases of MDR–TB were notified. By the end of 2008, 55 countries and territories had reported at least one case

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of extensively drug-resistant TB (XDR–TB) which are defined as MDR strains that are also resistant to a fluoroquinolone (FQ) and at least one second-line injectable agent (amikacin (AM), Kanamycin (KM) and/or capreomycin (CM) [1].

Fluoroquinolones are broad-spectrum antimicrobial agents that have been used with increasing frequency over the past decade. The particular advantages of fluoroquinolones are their high bioavailability, convenient dosing intervals, and efficacy against a wide array of bacterial infections, including community-acquired pneumonia [2,3].

Fluoroquinolones—in particular, levofloxacin, gatifloxacin, and moxifloxacin—have in vitro and in vivo activity against *Mycobacterium tuberculosis* [4,5]. Fluoroquinolone resistance in patients with newly diagnosed cases of tuberculosis would be important if identified, because of the current role of fluoroquinolones in the treatment of tuberculosis (i.e., treatment of patients resistant to or intolerant of first-line therapy) and the potential of this class of drugs to become first line therapy [6,7].

However, resistance to fluoroquinolones in cases of tuberculosis is not routinely assessed, particularly in isolates that are susceptible to current first-line agents.

Current Infectious Diseases Society of America/American Thoracic Society guidelines for community acquired pneumonia recommend that fluoroquinolones be used for both inpatient and outpatient treatment of pneumonia [8].

As a result, fluoroquinolones are frequently prescribed to people who are subsequently diagnosed with tuberculosis.

Among a cohort of patients with tuberculosis in Tennessee from 2000 to 2004, 23% received fluoroquinolone monotherapy before diagnosis. The proportion of exposed people increased from 9% in 2000 to 41% in 2004 (P, 0.001) [9].

The cellular target of FQs in *M. tuberculosis* is DNA gyrase, a type II topoisomerase consisting of two A and two B subunits encoded by gyrA and gyrB genes, respectively [10].

Mutations in a small region of gyrA, called quinolone resistance-determining region (QRDR) and, less frequently, in gyrB are the primary mechanism of FQ resistance in *M. tuberculosis* [11,12].

Analysis of QRDR alone by genotypic tests has been suggested as sufficient for rapid identification of vast majority of FQ-resistant *M. tuberculosis* strains as additional target of gyrB did not enhance the sensitivity significantly [13,14].

Reports show that the majority (approximately 50–90%) of FQ-resistant MTB isolates carry mutations in the quinolone resistance-determining region (QRDR) of the gyrA gene [14], and that a small number have mutations in the gyrB gene [15,16]. It was previously postulated that efflux pump mechanisms account for FQ resistance in isolates with wild-type gyrAB genes [17].

Fluoroquinolone resistance in *M. tuberculosis* can develop after as little as 13 days of fluoroquinolone therapy [10]. Although fluoroquinolone resistance in *M. tuberculosis* is not routinely assessed, the proportion of newly diagnosed (i.e., previously untreated patients with tuberculosis with fluoroquinolone resistance has ranged from 0.15% to 3.6% in previous reports [18,19].

Extensive use of fluoroquinolones for treatment of bacterial infections might result in primary fluoroquinolone-resistant tuberculosis. If primary resistance became common, this would negate the potential of fluoroquinolones to become part of first-line tuberculosis treatment. PCR-based techniques provide new possibilities for the rapid diagnosis of first- and second-line drug resistance, however, not all mycobacterial laboratories have access to DNA sequencing facilities [20].

Therefore, we evaluated the rate of fluoroquinolone resistance among *M. tuberculosis* isolates.

Materials and methods

Patient population

We conducted a cohort study of adult patients (age >18 years) with sputum positive, culture confirmed pulmonary tuberculosis from both El Abassia & El Omrania Chest hospitals in the period between September 2011 and February 2012. This study was approved from ministry of health tuberculosis control program.

Early-morning sputum specimens were collected and transported to the clinical laboratory for smear examination.

Detailed history taking was obtained from patients as regards previous history of anti tuberculous treatment, recent antibiotic used especially fluroquinolone.

Sample collection & transport

The patient was instructed to cough deeply and expectorated sputum specimens were collected from all patients in a screw-cap, leak-proof sterile containers. Specimens were handled with care as regards collection and transportation.

Decontamination and concentration

The initial concentration of NaOH is 4%. This 4% NaOH solution is mixed with an equal quantity of sodium citrate solution (2.9%) to make a working solution (NaOH concentration in this solution is 2%). When an equal quantity of NaOH–NALC–citrate and sputum are mixed, the final concentration of NaOH in the specimen is 1% [21]. Transfer the specimen to a 50 ml centrifuge tube with a screw cap.

Add NaOH–NALC–sodium citrate solution in a volume equal to the quantity of specimen. Tighten the cap.

Vortex lightly or hand mix for about 15–30 s. Invert the tube so the whole tube is exposed to the NaOH–NALC solution.

Wait 15–20 min (up to 25 min maximum) after adding the NaOH–NALC solution. Vortex lightly or hand mix/invert every 5–10 min or put the tubes on a shaker and shake lightly during the whole time.

Make sure the specimen is completely liquefied. If still mucoid, add a small quantity of NALC powder (30–35 g) directly to the specimen tube. Mix well.

At the end of 15–20 min, add phosphate buffer (pH 6.8) up to the top ring on the centrifuge tube (plastic tube has a ring for 50 ml mark). Mix well (lightly vortex or invert several times). Addition of sterile water is not a suitable alternative for the phosphate buffer.

Centrifuge the specimen at a speed of 3000*g* or more for 15–20 min. Use of refrigerated centrifugation at a higher speed is known to increase recovery of mycobacteria 2, 62.

After centrifugation, allow tubes to sit for 5 min to allow aerosols to settle. Then carefully decant the supernatant into

a suitable container containing a mycobactericidal disinfectant. Make sure the sediment is not lost during decanting of the supernatant fluid. Add a small quantity (1–2 ml) phosphate buffer (pH 6.8) and resuspend the sediment with the help of a pipette or vortex mixer.

Use the resuspended pellet for making smears and for inoculation of MGIT tubes [22]. Ziehl–Neelsen staining of *M. tuberculosis* was done according to standards [23].

Culture

Culture was done using Mycobacteria Growth Index Test (MGIT).[24].

Molecular detection of gyrA & gyrB was done according to Soudani et al. [25].

DNA extraction

Colony was taken from MGIT Template DNA was prepared by QIAGEN QIAmp DNA mini kit. Positive control was used *Escherichia coli*, *Staphylococcus aureus* resistant to ciprofloxacin.

Amplification

Using Perkin Elmer 9700 for gyrA, a DNA fragment of 216 bp, corresponding to the QRDR, was generated by PCR with the primer pair Pri9 (5¢-CGCCGGGTGCTCTATG-CAATG-3¢) and Pri8 (5¢-CGGTGGGTCATTGCCTGGC-GA-3¢), used at 4 lM. Amplification reactions were performed as previously described by [26]. For gyrB, a 322-bp fragment was amplified using the primer pair gyrBA (5¢-GAGTTGGTGCGCGCTAAGAGC-3¢) and gyrB E (5¢-CGGCCATCAGCACGATCTTG-3¢) at 0.4 lM. Amplification reactions were performed as previously described by Dauendorffer et al. [27].

Detection of (QRDR) by PCR

Amplified products were subjected to electrophoresis in 2% agarose gels in Tris–borate–EDTA buffer and visualized under UV light. The gyrA band was visualized at 216 bp and gyrB at 322 bp.

Results

There were 40 consecutive adults, who had culture confirmed pulmonary tuberculosis during the study period.

Six Female (15%) & 34 (85%) males

Mean age 47.1 years (32–72 years)

Twenty eight (70%) newly diagnosed (patients were deemed to have newly diagnosed tuberculosis if they had not received standard antituberculosis treatment before developing culture-confirmed tuberculosis).

Twelve relapse (30%)

Twelve patients were associated with risk factors for lower immunity (9 patients (22.5%) were diabetics, and 3 patients (7.5%) were drug addict).

All cases had chest X-ray findings (infiltration, cavitation). (See Fig. 1, Tables 1 and 2).

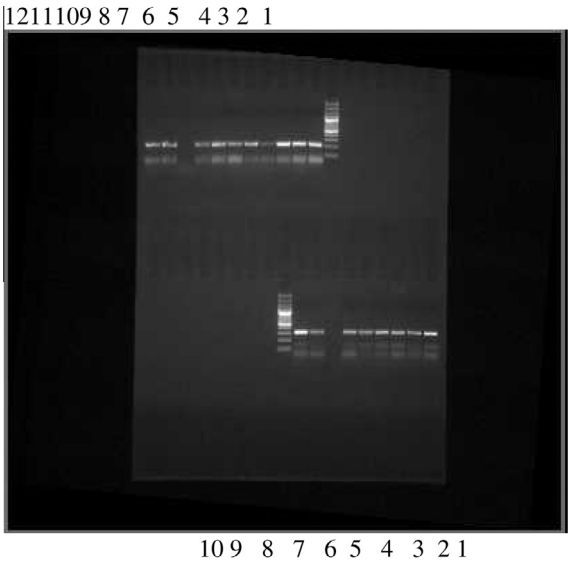

Figure 1 The above gel is for gyrA the (ladder) used is from 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 till 2000 and gyrA is at 216 bp, LAN 1 from the right side is for the marker (ladder), LAN 2–9, 11 and 12 are positive for gyraA, and the gel below is for gyrB. gyrB is at 322 bp, the marker (ladder) used is from 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000 till 2000 from the right side LAN 1–6, 8 and 9 are positive for gyra B, LAN 10 is for the ladder. (This photo is taken by gel documentation system).

Table 1 Clinical data of patients selected.

Sex	34 Male	6 Female
Age	18–72 years old	Mean age 47.1
Cases	28 New cases (70%)	12 Relapse (30%)
Risk factors	9 Diabetics 4 addicts one on steroids (IPF)	

Table 2 Laboratory findings.

Zeil Neelsen for Sputum −ve and MGIT −ve	3
Zeil Neelsen for Sputum −ve and MGIT +ve	1
Zeil Neelsen for Sputum +ve and MGIT +ve	34
Zeil Neelsen for Sputum +ve and MGIT −ve	3
Only gyrA +ve	7(17.5%)
Only gyrB +ve	5(12.5%)
Both gyrA and gyrB +ve	22(55%)
Both gyrA and gyrB −ve	6(15%)
gyrA +ve	29(72.5%)
gyrB+ve	28(70%)

Mutations were observed in the QRDRs of both gyrA and gyrB in 22 isolates (55%). Only gyrA +ve in 7(17.5%) isolates. Only gyrB +ve in 5(12.5%) isolates. Total gyrA +ve in 29(72.5%) and total gyr B +ve in 28(70%) isolates. Both gyrA and gyrB −ve in 6(15%).

Discussion

This study on fluoroquinolone resistance among smear-positive pulmonary TB cases is the first of its kind in Egypt.

Fluoroquinolones (FQs) are the most promising antituberculous therapeutic agents to be developed in 40 years [28].They are widely used for the treatment of multidrug-resistant (MDR) tuberculosis (TB) despite the lack of clinical trials evaluating optimal doses, duration, and combinations [29]. There is concern about levels of preexisting FQ-resistant TB in regions with high drug resistance rates because these drugs are often available over the counter and are additionally prescribed as broad-spectrum antibiotics for the treatment of undiagnosed respiratory infections [30].

Mutations in short regions of gyrA, known as QRDR, have been associated with FQ resistance in MTB [31]. Several studies [32] have analyzed the mutations in the gyrA gene in clinical isolates of MTB. Most of these studies focused on the frequency of the mutations in gyrA/gyrB genes in FQ-resistant MTB strains. Thereare, however, no data on the association of mutations in gyrA/gyrB and FQ resistance levels in MTB isolates.

Only Yin et al. have shown conclusively that different substitutions of amino acid 94 resulted in different levels of levofloxacin resistance [33].

Although the sample size in our study was substantially small, the incidence of infection with fluoroquinolone-resistant *M. tuberculosis* appeared to be high. This may have been due at least in part to increased use of fluoroquinolones for the treatment of lower respiratory tract infection. One rather important mechanism for the development of fluoroquinolone-resistant TB is the suboptimal use of second line drug regimens, especially in the presence of a poorly functioning program in the treatment of multidrug-resistant TB.

In our study 55% of the clinical isolates were positive for both gyrA and gyrB, this agree with Yin and Yu [32] as they detect 44 of 60 (73.3%) levofloxacin-resistant MTB clinical isolates, including 17 of 18 (94.4%) high-level resistant strains and 27 of 42 (64.3%) low-level resistant strains, had mutation in QRDR of gyrA gene.

The mutation patterns involved six patterns of single codon mutation (H70R, A90V, S91A, D94G, D94A or D94N) and one pattern of double codons mutation (A90V with D94A). Of 60 levofloxacin-resistant MTB clinical isolates, only one (1.6%) mutated in gyrB gene (T511N) [31].

Also agree with Zhenling et al. [34]. Mutations were observed in the QRDRs of gyrA/gyrB in 87 out of 95 (91.6%) OFX-resistant MTB strains [34].

Mutations other than those affecting gyrA and other mechanisms could result in FQ-R, including: decreased cell-wall permeability to drug, efflux pumps, drug sequestration or perhaps even drug inactivation [35]. In a small number of cases, FQ-R could be associated with gyrB mutations and a probable efflux mechanism [36].

Our study was limited by the lack of the patients fluroquinolone exposure data. There is also natural polymorphism in the nucleotide sequences of gyrA/gyrB, that is not associated with drug resistance as natural polymorphism occur in both fluoroquinolone sensitive and resistant bacteria, so we recommend performing DNA sequencing and concomitant drug susceptibility testing.

Conclusion

The incidence of FO-resistant *M. tuberculosis* is gradually increasing to alarming levels this may be due to wide spread use of this vital groups of drugs in community-acquired pneumonia and urinary tract infections.

Recommendations

We recommend judicious use of fluoroquinolones as a broad spectrum antibiotics and it is ideally to be reserved for treatment of resistant TB or at least limit the use of prolonged or repeated courses of FQ in patients at risk of having active TB.

We recommend further researches as DNA sequencing to exclude natural polymorphisms and detect different kinds of mutations affecting the genes. Also, recommend drug sensitivity testing to correlate the degree of resistance and drug inhibition with gene mutation and the possible methods to overcome this problem.

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